Journal of Chemical Ecology, Vol. 21, No. 11, 1995

ATTRACTANTS FROM *Staphylococcus aureus* CULTURES FOR MEXICAN FRUIT FLY, *Anastrepha ludens*

D.C. ROBACKER[1,*] and R.A. FLATH[2]

[1]*Crop Quality and Fruit Insects Research, ARS, USDA*
2301 South International Blvd., Weslaco, Texas 78596
[2]*Plant Protection Research, ARS, USDA*
800 Buchanan, Albany, California 94710

(Received February 28, 1995; accepted July 12, 1995)

Abstract—Volatile chemicals from tryptic soy broth cultures of *Staphylococcus aureus* that attract sugar-fed, protein-hungry adult Mexican fruit flies were identified. Chemicals identified from the headspace above the filtrate of the bacterial cultures were ammonia, trimethylamine, isoamylamine, 2-methylbutylamine, 2,5-dimethylpyrazine, and acetic acid. Each chemical attracted flies. A mixture of the chemicals in the same concentrations as were found in the bacterial filtrate was 89% as effective in attracting flies as the bacterial filtrate in laboratory bioassays. Additional chemicals were identified from various concentrated or pH altered preparations made from the filtrate. Many of these chemicals also attracted flies. One of these chemicals, dimethylamine, was the most effective chemical identified. The use of solid-phase microextraction for volatile collection and of thick-film (5-μm) capillary GC columns was essential to the success of this work.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, bacteria, amines, solid-phase microextraction.

INTRODUCTION

Associations of bacteria with fruit flies have been known for nearly a century (Petri, 1910) but remain little understood to this day. Popular views during the first half of this century were that bacteria and fruit flies engaged in obligate symbioses (Stammer, 1929; Hellmuth, 1956; Boush and Matsumura, 1967; Miyazaki et al., 1968). Later studies suggested that bacteria serve as a protein

*To whom correspondence should be addressed.

0098-0331/95/1100-1861$07.50/0

source for fruit flies (Drew and Lloyd, 1989; Robacker and Moreno, 1995). Neither of these views is entirely satisfactory nor entirely disproven, and the possibility of other explanations must also be acknowledged. Whatever the explanation, associations of bacteria with fruit flies seem nearly ubiquitous. As examples, Stammer (1929) isolated bacteria from 37 species of fruit flies and Hellmuth (1956) from 43 species. Fitt and O'Brien (1985) isolated at least 20 species of bacteria from four *Dacus* species, and Martinez et al. (1994) isolated at least 11 species of bacteria from *A. ludens* alone.

Some understanding of the nature of fruit fly–bacteria associations has been gained by studies showing that bacteria or their metabolic products attract fruit flies in many cases. Several studies have shown that inoculation of protein baits with bacteria increased the attractiveness of the baits (Gow, 1954; Bateman and Morton, 1981; Drew and Fay, 1988). Others have shown that bacteria or their cultures alone, or combined with fruit volatiles, make good trap baits (MacCollom et al., 1992; 1994; Martinez et al., 1994) or attract fruit flies in laboratory bioassays (Jang and Nishijima, 1990; Robacker et al., 1991; MacCollom et al., 1992).

Papers in which attractants from bacterial preparations have been assessed generally reported little progress beyond identification and evaluation of ammonia (Gow, 1954; Drew and Fay, 1988). Most who have worked with bacterial attractants agree that additional chemicals probably are involved. Drew (1987) tested several components of bacterial odor and demonstrated that one of these, 2-butanone, was significantly more attractive to *Bactrocera tryoni* Froggatt than cuelure, the standard attractant for this fly. The attractiveness of this chemical was attributed to its structural similarity to cuelure rather than any bacteria–fruit fly relationship. Two other components, butanol and isopentanol, were not attractive.

Recent work by Robacker et al. (1993) showed that standard insect attractant volatile collection methods were unsuitable for collecting attractive compounds from a highly attractive bacterial fermentation. Specifically, volatile collections of bacterial odors on adsorbents typically used in insect attractant research such as Porapak Q, Tenax, and Super Q were not attractive to Mexican fruit flies. The work also demonstrated that the attractants were water soluble and could not be partitioned into organic solvents by solvent–solvent extraction. The conclusion of the work was that the most attractive chemicals probably were low-molecular-weight amines or other compounds containing ionizable nitrogen. It also appeared that some attractants did not have properties of amines and possibly were carboxylic acids or other water-soluble organic molecules. These findings may help explain the lack of progress in identification of bacteria-produced chemicals attractive to fruit flies since most reported studies used standard volatile collection methods.

The current reseach is a continuation of the work reported by Robacker et al. (1993) to identify the volatile chemicals produced by tryptic soy broth cultures of *Staphylococcus aureus* (strain RGM-1) originally isolated from the mouthparts of a laboratory-colony, adult Mexican fruit fly (Robacker et al., 1991). Solid-phase microextraction technology developed especially for analysis of trace organics in aqueous solution (Belardi and Pawliszyn, 1989; Zhang and Pawliszyn, 1993) was used to collect volatiles from the headspace above the filtrate of RGM-1 cultures and various concentrated or pH-altered preparations made from the filtrate. Trapped chemicals were identified and tested for attractiveness to Mexican fruit flies.

METHODS AND MATERIALS

Insects and Test Conditions. Flies used to test attractiveness of bacterial preparations and chemicals identified from the preparations were from a culture that had been maintained on laboratory diet for about 400 generations with no wild-fly introductions. Mixed-sex groups of 180–200 flies were kept in 473-ml cardboard cartons with screen tops until used in tests. Flies were tested when 5–12 days old. Flies were deprived of protein as adults but were fed sucrose and water up until the time of attractiveness testing. Recent tests have shown that protein-deprived flies are maximally attracted to bacterial odor when 5–7 days old and remain highly responsive through 15 days of age (Robacker and Garcia, 1993). All tests were conducted in the laboratory between 08:30 and 15:30 hr under a combination of fluorescent and natural light. Laboratory conditions were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 06:30 to 19:30 hr.

Bacterial Preparations. S. aureus strain RGM-1 (Robacker et al., 1991) was grown in tryptic soy broth (Difco Laboratories, Detroit, Michigan) in a shaker for 144 hr at 30°C. Bacterial culture was centrifuged at 10,000 rpm for 20 min and separated into pellet and supernatant. The pellet was discarded because previous experiments indicated that the pellet, which contained most of the bacterial cells, was less attractive than tryptic soy broth (Robacker et al., 1993). Supernatant was filtered through 0.45-μm type HA aqueous filters (Millipore Corporation, Bedford, Massachusetts) followed by 0.22-μm type GV aqueous/organic filters (Millipore). Filtration was conducted to remove remaining bacterial cells. Robacker et al. (1993) demonstrated that the attractants were dissolved in the filtrate.

RGM-1 filtrate was concentrated and otherwise manipulated to enhance collection and identification of volatiles. RGM-1 concentrate was prepared by lowering pH of filtrate to 4.0 with 10 N HCl and concentrating it 12-fold by

rotary evaporation (Rotavapor RE-111, Buchi-Brinkman Instruments, Inc., Westbury, New York). The evaporation was aided by heating the filtrate while pulling a vacuum on the system. RGM-1 salt was prepared as described previously (Robacker et al., 1993), with a few differences. Briefly, RGM-1 filtrate was put into a two-neck 500-ml flask and heated gently while steam was used to sweep the headspace from the flask into a water-cooled condenser. Steam was generated from vigorous boiling of Milli-Q (Millipore)-grade water in a second two-neck 500-ml flask. The procedure was carried out until 400 ml of steam distillate was collected per 100 ml of original RGM-1 filtrate. The pH of the steam distillate was lowered to 4.0 with HCl, and the water was removed by rotary evaporation as described for the RGM-1 concentrate. The resulting dry powder was termed RGM-1 salt.

Volatile Collections and Chemical Identifications. Chemicals in the headspace above RGM-1 filtrate, RGM-1 filtrate adjusted to pH 11 with aqueous NaOH, RGM-1 concentrate at pH 4 and also pH 11 after adjustment with NaOH, and RGM-1 salt partially dissolved in a few drops of saturated aqueous NaOH were collected and identified. Volatiles were collected by solid-phase microextraction (SPME) with a 100-μm polydimethylsiloxane-coated fiber (Supelco, Inc., Bellefonte, Pennsylvania). The fiber was inserted through a septum into the headspace above 1 ml of bacterial preparation in a 4-ml vial for 5 min to 24 hr. Sampling time varied inversely with the concentration of chemicals in the preparation.

Volatiles also were collected on ORBO 52 silica gel tubes (Supelco) connected to an outlet port in the volatile collection apparatus developed by Heath and Manukian (1992). One half milliliter of saturated NaOH was added to 0.5 ml of RGM-1 concentrate in a 4-ml glass vial to produce free amines. The vial was mounted inside the apparatus such that the inlet end of the silica tube was inside the lip of the vial. Air was pulled through the system for 17 hr at 0.5 liters/min. The silica gel was removed from the tube and extracted by sonication in a 4-ml glass vial with 0.5 ml of methanol for 0.5 hr followed by a rinse with a second 0.5 ml of methanol. The two methanol aliquots were combined and filtered through 0.45-μm type HV syringe filters (Millipore).

GC analyses were conducted on DB-1 and DB-5 fused-silica capillary columns (J & W Scientific, Folsom, California). The DB-1 column (60 m, 0.32 mm ID, 5 μm film) was used for most analyses because it appreciably retained but did not irreversibly adsorb nanogram-level quantities of ammonia and C_1 to C_5 aliphatic amines, providing baseline resolution of most of these compounds. The DB-5 column (30 m, 0.53 mm ID, 5 μm film) was used to separate a few amines that coeluted on the DB-1 column and to confirm identifications that were based on retention time matching to standards on the DB-1 column. Preliminary work with films of less than 5 μm demonstrated that the thick films (5 μm) were responsible both for the lack of irreversible adsorption and appreciable

retentions of the chemicals discussed above and also improved peak symmetry allowing for better resolution of components with similar retention times.

GC analyses were conducted with a Shimadzu GC-9A (Shimadzu Scientific Instruments, Inc. Columbia, Maryland) with flame ionization and flame thermionic (model FTD-9) detectors. The FTD was used to establish the presence of C—N bonds and thus confirm characterization of many of the bacteria volatiles as amines, to provide highly sensitive detection of trace quantities of several of the compounds and to calculate FID/FTD response ratios of unknowns for comparison with ratios of standards as another identification method.

Volatiles were thermally desorbed from the SPME fiber in the split/splitless injection port at 210°C. Splitless injection was conducted with the splitter closed 0.5 min. Analyses were at 100°C. ORBO 52 extracts were concentrated 10-fold under nitrogen assisted by low heating. Splitless injection was also used for 1 μl samples of the concentrated ORBO 52 extracts. Analyses of ORBO 52 extracts were conducted with an initial temperature at 100°C for 5 min then 4°C/min to 210°C. For all analyses, carrier gas was helium at a linear velocity of 20 cm/sec.

GC-MS analyses were conducted with a Finnigan MAT 4500 GC-MS data system (Finnigan MAT, San Jose, California) operated in the electron-impact ionization mode (emission current = −0.30 mA, electron energy = 70 eV, multiplier voltage = −1150 V). Mass spectral data were acquired over a mass range of 33–350 amu, using a 1-sec scan rate. Volatile injection was the same as described above except the injector temperature was 220°C and the SPME fiber was exposed in the injector for 2 sec. The column-oven temperature was programmed from 50 to 200°C at 4°/min. Carrier gas was helium at 28 cm/sec.

Cage-Top Bioassays. Various RGM-1 preparations and chemicals identified from the preparations were tested for attractiveness using cage-top bioassays. Tests of bacterial preparations were conducted because volatiles were identified from many of these preparations, and we wanted to compare attractiveness of preparations based on similarity of their volatiles.

Bioassays were conducted by placing four filter paper triangles (3 cm/side), two containing test samples and two containing appropriate controls, near the corners on the top of an insect cage (30 cm/side, aluminum-screened). The numbers of flies beneath each filter paper were counted once each minute for 10 min. The two papers containing test chemical were positioned diagonally from each other on two corners of the cage top, and the two papers containing controls were positioned diagonally from each other on the other two corners. The filter papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction and not contact chemoreception was solely responsible for the response of the flies. One carton of 180–200 flies was used in each bioassay cage.

For bioassays of RGM-1 preparations, 10-μl test quantities were put on two papers and 10 μl of water were put on two papers as controls. The ORBO 52 collections were bioassayed similarly except methanol was used as the control. Ten replications were conducted for each RGM-1 preparation and for the ORBO 52 collection.

Chemicals identified from various RGM-1 preparations were obtained (Table 1) and dissolved in water or methanol for attractiveness testing. Chemicals dissolved in water were: dimethylamine HCl; trimethylamine HCl; ethylamine; propylamine; isopropylamine; isobutylamine; isoamylamine; 2-ethyl-5-methylpyrazine; and acetic acid. One drop of saturated NaOH was added to solutions of dimethylamine HCl and trimethylamine HCl to produce free amines.

TABLE 1. CHEMICAL NAMES, SOURCES, AND PURITIES OF CHEMICALS USED AS GC STANDARDS AND/OR TESTED IN CAGE-TOP BIOASSAYS

Chemical	Source[a]	Purity (%)[b]
Ammonium bicarbonate	Sigma	99
Ammonium carbonate	Aldrich	unknown[c]
Methylamine HCl	Sigma	99
Dimethylamine HCl	Sigma	>95
Trimethylamine HCl	Sigma	99
Ethylamine (70% in water)	Sigma	>99
Diethylamine	Aldrich	98
Propylamine	Aldrich	99
Isopropylamine	Aldrich	99
Butylamine	Sigma	99
Isobutylamine	Aldrich	99
sec-Butylamine	Aldrich	99
tert-Butylamine	Aldrich	98
Isoamylamine	Aldrich	99
2-Methylbutylamine	Aldrich	>97
2,5-Dimethylpyrazine	Aldrich	99
2-Ethyl-5-methylpyrazine	Pyrazine Specialties	50[d]
Acetamide	Aldrich	>99
Propionamide	Aldrich	97
2-Methylpropionamide	Aldrich	99
Acetic acid	Mallinckrodt	>99
Methyl disulfide	Aldrich	99

[a] Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; Sigma Chemical Company, St. Louis, Missouri; Pyrazine Specialties, Inc., Atlanta, Georgia; Mallinckrodt Specialty Chemicals Co., Paris, Kentucky.
[b] Determined from label information and GC analysis.
[c] Mixture with ammonium bicarbonate and ammonium carbamate.
[d] Approximately equal mixture with 2-ethyl-6-methylpryazine.

The other chemicals were dissolved in methanol. Four concentrations of each chemical were prepared and subsequently four quantities (10 ng, 100 ng, 1 μg, and 10 μg of most chemicals; 100 ng, 1 μg, 10 μg, 100 μg of amides) of each chemical were tested, each in 10 μl of solution. The control for each chemical was 10 μl of water or methanol for chemicals dissolved in water or methanol, respectively. Ten replications were conducted for each of the four concentrations of all of the chemicals, except trimethylamine and 2,5-dimethylpryazine for which 20 replications were conducted.

Chemicals identified from headspace above RGM-1 filtrate that were attractive to flies in cage-top bioassays were mixed together in aqueous solution in the same concentrations as were found in RGM-1 filtrate. Concentrations of the chemicals in RGM-1 filtrate were determined by comparing SPME collections in headspace above known concentrations of mixtures of standards, with SPME collections in RGM-1 headspace. The pH of the standards solution was adjusted to 7.9 with aqueous NaOH to equal the pH of unaltered RGM-1 filtrate. A trial-and-error approach was used in which standards were mixed together in a vial in various concentrations that were adjusted until FID peak heights on the DB-1 column matched those obtained from a vial containing RGM-1 filtrate. Concentrations in the final synthetic mixture that matched RGM-1 were: ammonium carbonate, 2.0 mg/ml; trimethylamine HCl, 4.0 μg/ml; isoamylamine, 1.6 μg/ml; 2-methylbutylamine, 0.3 μg/ml; 2,5-dimethylpyrazine, 5.0 μg/ml; and acetic acid, 250 μg/ml. Sources of chemicals used in the mixture are shown in Table 1. The final synthetic mixture was tested in cage-top bioassays using water as the control. Test quantities were 10 μl. Twenty-eight replications were conducted. Separate bioassays of RGM-1 filtrate ($N = 28$) were conducted concurrently using flies from the same cohort.

Statistical Analysis. Responses of flies to the various bacterial preparations were compared with each other in a randomized complete block experiment. This was done by subtracting total counts at solvent-blank papers from total counts at treatment papers to obtain differences that were then subjected to analysis of variance. Means were compared by Fisher's protected least significant difference method. Total counts of flies at filter papers containing test samples were compared to counts at solvent-blank papers by paired t tests in all experiments. Finally, responses of flies to the synthetic mixture of RGM-1 chemicals were compared with responses to RGM-1 filtrate by a t test of independent samples. Data for this test were differences between total counts at treatment and solvent-blank papers for each replication.

RESULTS AND DISCUSSION

All bacterial preparations were more attractive to flies than water in cage-top bioassays (Table 2). RGM-1 filtrate was the most attractive preparation.

TABLE 2. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING WATER OR VARIOUS BACTERIAL PREPARATIONS USING CAGE-TOP BIOASSAYS

	Preparation[a]	Water
RGM-1 filtrate	141.7 a	14.3
RGM-1 filtrate pH 11	108.2 b	14.5
RGM-1 concentrate pH 4	29.9 c	18.5
RGM-1 concentrate pH 11	99.2 b	17.4
RGM-1 salt pH 11	113.5 b	17.1
ORBO 52 volatiles collection	36.4 c	20.1

[a] Mean responses to all bacterial preparations were significantly greater than responses to water by paired *t*-tests ($P < 0.05$); mean responses to bacterial preparations followed by the same letter were not significantly different from each other by LSD ($P < 0.05$).

Most of the results in Table 2 are similar to findings reported in Robacker et al. (1993). One preparation not tested by Robacker et al. (1993) was the ORBO 52 volatile collection. Here we show that at least some of the material responsible for attractiveness can be trapped on ORBO 52 silica gel tubes, although the attractiveness was low compared with that of RGM-1 filtrate. Robacker et al. (1993) reported that attractants evolving from concentrated RGM-1 filtrate could not be trapped on Porapak Q, Super Q, or Tenax GC.

Chemicals identified from headspace of RGM-1 filtrate at pH 7.9 (unaltered) are shown in Table 3. Calculated concentrations (mean ± SE; $N = 3$) in RGM-1 filtrate were: ammonia, 710 ± 41 μg/ml; trimethylamine, 2.5 ± 0.1 μg/ml; isoamylamine, 1.6 ± 0.7 μg/ml; 2-methylbutylamine, 0.3 ± 0.05 μg/ml; 2,5-dimethylpyrazine, 5.0 ± 0.1 μg/ml; and acetic acid, 250 ± 84 μg/ml.

All six chemicals found in the headspace above the RGM-1 filtrate attracted Mexican fruit flies. Data for trimethylamine, isoamylamine, 2-methylbutylamine, 2,5-dimethylpyrazine, and acetic acid are shown in Table 4. Ammonia was tested previously, and the data were reported in Robacker and Warfield (1993). Like most of the chemicals, ammonia was most attractive at the higher concentrations. Ammonia, isoamylamine, 2-methylbutylamine, and acetic acid appear to be nearly equally attractive according to ratios of counts at chemicals (10 μg test quantities) to counts at water. Trimethylamine and 2,5-dimethylpyrazine were much less attractive than the others. Trimethylamine was unusual in that it was attractive only at the lower concentrations.

The synthetic mixture of the chemicals found in RGM-1 filtrate was much more attractive than water but significantly less attractive than RGM-1 filtrate (Table 5). Nevertheless, the response to the synthetic mixture was 89% as high as the response to RGM-1 filtrate, indicating that the synthetic mixture was a fairly good match to the RGM-1 filtrate. Reasons for the slightly lower response

TABLE 3. CHEMICALS IDENTIFIED FROM VARIOUS BACTERIAL PREPARATIONS

	Chemicals Identified and Methods of Identification[a]
RGM-1 filtrate (pH not altered from 7.9)	ammonia, 2-methylbutylamine by DB-1 RT, FID/FTD ratios; trimethylamine, isoamylamine, 2,5-dimethylpyrazine, acetic acid by MS, DB-1 RT, FID/FTD ratios
RGM-1 filtrate (pH 11)	ammonia, trimethylamine, isoamylamine, 2-methylbutylamine by DB-1 RT, FTD detection; 2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine by MS
RGM-1 concentrate (pH 4)	acetic acid by MS
RGM-1 concentrate (pH 11)	ammonia, 2-methylbutylamine by DB-1 RT, FTD detection; trimethylamine, isoamylamine by MS, DB-1 RT, FTD detection; acetic acid, methyl disulfide by MS
RGM-1 salt (NaOH added)	ammonia, methylamine, dimethylamine, trimethylamine ethylamine, diethylamine, propylamine, isopropylamine, butylamine, isobutylamine, *sec*-butylamine, *tert*-butylamine, isoamylamine, 2-methylbutylamine by both DB-1 and DB-5 RT, FID/FTD ratios
ORBO 52	acetamide, propionamide, 2-methylpropionamide, 3-methylbutyramide by MS

[a] MS = mass spectrometry using DB-1 column; RT = retention time comparison with standards on DB-1 and/or DB-5 columns; FID/FTD ratios = comparison of response by flame ionization vs flame thermionic detectors

to the synthetic mixture are unknown. Possible reasons are: (1) one or more attractants in RGM-1 filtrate was not identified and consequently left out of the synthetic mixture; (2) contaminants in the synthetic mixture from the commercially obtained chemicals were repellent; and/or (3) concentrations of chemicals in the synthetic mixture were not identical to concentrations in RGM-1 filtrate.

The synthetic mixture did not have the same odor as RGM-1 filtrate, as judged by human olfaction. The Porapak Q and similar volatile collections tested in earlier work (Robacker et al., 1993) smelled very similar to RGM-1 filtrate. Nevertheless, the Porapak Q volatile collections did not attract Mexican fruit flies, whereas the synthetic mixture used in the present study was highly attractive.

Chemicals identified from RGM-1 filtrate adjusted to pH 11 were ammonia, trimethylamine, isoamylamine, 2-methylbutylamine, 2,5-dimethylpyrazine, and 2-ethyl-5-methylpyrazine (Table 3). Only acetic acid was identified from RGM-1 concentrate at pH 4. No other carboxylic acids were detected. Chemicals identified from RGM-1 concentrate adjusted to pH 11 were ammonia, trimethylamine, isoamylamine, 2-methylbutylamine, acetic acid, and methyl disulfide.

TABLE 4. MEAN COUNTS OF *A. ludens* AT SOLVENT BLANKS OR TEST CHEMICALS IDENTIFIED FROM VARIOUS BACTERIAL PREPARATIONS USING CAGE-TOP BIOASSAYS

	10 ng		100 ng		1 μg		10 μg	
	Test	Solvent	Test	Solvent	Test	Solvent	Test	solvent
Dimethylamine[a]	13.3	11.5	15.4	14.0	41.3[b]	12.8	77.0[b]	12.3
Trimethylamine[a]	19.2[b]	13.1	22.1[b]	16.0	19.1	16.6	19.3	17.8
Ethylamine	13.1	13.1	12.5	12.6	44.8[b]	15.2	43.8[b]	13.4
Diethylamine	14.8	14.8	14.3	15.0	20.3[b]	13.2	29.1[b]	11.1
Propylamine	13.0	13.3	15.8	10.4	27.3[b]	14.2	28.8[b]	12.3
Isopropylamine	12.9	13.9	12.4	10.0	25.5[b]	10.9	44.5[b]	12.0
Butylamine	12.8	10.7	16.4	13.5	32.3[b]	13.2	29.2[b]	15.2
Isobutylamine	10.7	10.2	14.1	11.2	28.8[b]	12.8	29.6[b]	12.1
sec-Butylamine	13.2	13.7	11.7	12.7	39.8[b]	12.7	29.6[b]	11.9
tert-Butylamine	17.5	17.7	20.4	16.3	20.2	17.7	25.8[b]	17.8
Isoamylamine	11.9	13.6	13.6	14.5	21.9[b]	10.7	43.4[b]	15.9
2-Methylbutylamine	20.6	16.1	20.8	21.4	23.8	23.1	36.6[b]	20.8
2,5-Dimethylpyrazine	14.3	15.4	16.0	15.6	18.9[b]	13.6	13.0	14.2
2-Ethyl-5-methylpyrazine	24.1	21.8	22.2	21.0	13.0[b]	18.9	8.9[b]	22.5
Acetic acid	15.9	10.1	14.5	12.2	21.8[b]	10.8	47.1[b]	14.1
Methyl disulfide	21.2	19.9	22.8	20.4	15.1	19.0	21.4	18.8

[a]Tested as their respective HCl salts at pH > 10.
[b]The mean response to a chemical was significantly different from the response to water by paired *t*-test ($P < 0.05$).

TABLE 5. MEAN COUNTS (±SE) OF *A. ludens* AT FILTER PAPERS CONTAINING SYNTHETIC MIXTURE OF CHEMICALS IDENTIFIED FROM RGM-1 HEADSPACE COMPARED WITH COUNTS AT PAPERS CONTAINING RGM-1 FILTRATE OR WATER USING CAGE-TOP BIOASSAYS

	Test preparation	Water
Synthetic mixture	135.9 ± 4.8[a]	20.5 ± 1.2
RGM-1 filtrate	152.9 ± 5.5	19.7 ± 1.2

[a] The mean response to the synthetic mixture was significantly greater than the response to water by a paired t test ($P < 0.01$), and significantly lower than the response to RGM-1 filtrate by a t test ($P < 0.05$)

Neither 2-ethyl-5-methylpyrazine nor methyl disulfide was attractive to flies (Table 4). 2-Ethyl-5-methylpyrazine was slightly repellent at the two highest test quantities. Methyl disulfide previously was identified from volatiles of the bacterium *Proteus* by Hayward et al. (1977), but it had not been tested as a fruit fly attractant.

Many additional chemicals were identified from the headspace above the RGM-1 salt partially dissolved in NaOH (Table 3). All of the chemicals were attractive to flies, mostly at the higher test quantities (Table 4). Dimethylamine was the most attractive chemical identified from any of the bacterial preparations according to ratios of counts at chemicals to counts at water. Attractiveness of methylamine was about equal to ammonia in experiments reported previously (Robacker and Warfield, 1993).

The only chemicals identified from ORBO 52 collections were acetamide, propionamide, 2-methylpropionamide, and 3-methylbutyramide (Table 3). Of the three that were tested, none attracted the flies (Table 6). These chemicals

TABLE 6. MEAN COUNTS OF *A. ludens* AT SOLVENT BLANKS OR TEST CHEMICALS IDENTIFIED FROM ORBO 52 COLLECTION OF VOLATILES FROM RGM-1 CONCENTRATE AT pH > 10 USING CAGE-TOP BIOASSAYS[a]

	100 ng		1 μg		10 μg		100 μg	
	Test	Solvent	Test	Solvent	Test	Solvent	Test	Solvent
Acetamide	12.2	10.3	14.3	14.0	13.0	15.2	13.9	12.0
Propionamide	9.5	9.1	10.0	9.0	11.3	9.3	9.9	10.9
2-Methylpropionamide	12.0	13.0	12.5	13.3	14.2	15.1	10.2	11.0

[a] 3-Methylbutyramide also was identified from ORBO 52 collection, but was not available for testing. None of the chemicals were more attractive than blanks.

were tested at higher concentrations than the others because of their lower volatilities. Other chemicals that may have been present in the ORBO 52 extract and which may have accounted for its attractiveness (Table 2) probably were lost when it was concentrated for GC-MS analysis.

Not all of the chemicals in the various preparations were identified. The emphasis of the investigation was to identify chemicals that were water soluble and contained ionizable nitrogen, because chemical characterization studies had indicated that the chemicals most attractive to *A. ludens* fit this profile (Robacker et al., 1993). Most of the unidentified chemicals were minor components. However, some relatively large FID peaks present in the headspace of the RGM-1 salt were not identified because they were not detectable by FTD. One exception was a major unidentified peak in the headspace of the RGM-1 salt that produced a large FTD/FID ratio, indicating that it contained C-N. This peak had a retention time that matched that of 1,2-dimethypropylamine on the DB-1 column but not on the DB-5 column.

Another important point is that some of the chemicals identified in this study may have been artifacts of the harsh procedures used to concentrate the volatiles. The strong acids and bases used to manipulate pH may have catalyzed various hydrolyses and condensations involving water, alcohols, carboxylic acids, amines, etc., present in the original solution. Furthermore, headspace sweeping with steam was assisted with heating, and no attempt was made to remove oxygen from the system. Thus, numerous products not originally present in the RGM-1 filtrate may have formed. This probably occured the most in the RGM-1 salt, the product that was produced using the most rigorous treatment. Interestingly, all of the chemicals identified from RGM-1 salt headspace were attractive to the flies. Perhaps many of these same chemicals also form in natural fermentations where heat, water, and oxygen are also present.

The chemicals identified from the headspace of unaltered RGM-1 filtrate are considered natural products of bacterial metabolism of the tryptic soy broth. Therefore, only these chemicals were quantified and used to construct a synthetic mixture to mimic bacterial odor. The other bacterial preparations were not reproduced for attractiveness testing because they may have contained chemicals that were not present in the original culture. Quite possibly, many of the same chemicals identified in the various other preparations, including RGM-1 salt, may have been present in the RGM-1 filtrate but at concentrations too low to detect by our methods.

This is the first investigation to identify volatile chemicals produced by a bacterial culture attractive to an insect species, test the attractiveness of the chemicals to the insect, construct a mixture of the chemicals at the same concentrations as found in the bacterial preparation, and test the attractiveness of the mixture relative to the attractiveness of the bacterial preparation. As it has been shown that preparations made from bacterial fermentations can be highly

attractive to fruit flies in the field (Martinez et al., 1994), it is hoped that results of this work can contribute to development of better attractants for the Mexican fruit fly and perhaps other species of Tephritidae. However, it should be noted that the study was not entirely successful, as the synthetic mixture was only 89% as attractive as the bacterial filtrate.

Acknowledgments—We thank Maura Rodriquez, Marco Gomez, Cyndi Rodriquez, and Pravait Kaochoung (Office of Atomic Energy for Peace, Bangkhen, Bangkok, Thailand) for technical assistance; Sammy Ingle for insects; W. Haddon (USDA-ARS, Albany, California) for HPLC-MS analyses of preliminary bacterial preparations; and R.R. Heath (USDA-ARS, Gainesville, Florida) for GC-MS analyses of preliminary HPLC fractions and for use of his volatile collection apparatus for ORBO 52 analyses. This work was partially supported by a grant (89-0440) from the California Department of Food and Agriculture. Use of a product brand in this work does not constitute an endorsement by the USDA.

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